THE INFLUENCE OF PANTOTHENIC ACID ON GROWTH OF PROTOZOA

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INTRODUCTION

During the past few years Williams and his associates have been concerned with isolating from many sources substances which stimulate the multiplication of yeast. These investigations finally have resulted in the isolation of a substance to which the name, "pantothenic acid" has been given (Williams et al., 1933). This is an appropriate name because the acid has been found in tissues of representatives of nearly all the plant and animal phyla. It would seem that a substance of such universal occurrence must possess some fundamental biological significance.

Pantothenic acid exerts a pronounced stimulating effect on the growth of the "Gebrüde Mayer" strain of *Saccharomyces cerevisiæ*. Furthermore, it has many properties in common with vitamin G (B_2). Such striking characteristics make it an ideal substance for experimentation with other microörganisms, especially with certain Protozoa because they are more closely related to higher animals than are yeasts. Results of such investigations would be particularly interesting if pantothenic acid should prove to be identical with vitamin G (B_2). Reports in the literature concerning the effects of such substances on the growth of bacteria-free strains of Protozoa are completely lacking, and any evidence to show that Protozoa do or do not require vitamins in their normal nutrition would be of general as well as special interest.

The writer wishes to express his appreciation to Professor R. J. Williams for supplying the pantothenic acid, and also to Miss E. Swaine for counting the organisms in making the tests.

MATERIAL AND METHODS

In testing the effect of such a substance as pantothenic acid on the growth of a protozoan form, it is imperative that the cultures tested are free from other contaminating microörganisms. For that reason all the test cultures used in this investigation are bacteria-free. It was decided to test the effect of pantothenic acid on the growth of a ciliate, *Colpidium striatum*, and a phytomonad flagellate, *Hæmatococcus pluvi-*

alis. The first organism is definitely animal-like in its food requirements while the latter is plant-like in its nutrition. A comparative study was thus conducted with two remotely related protozoan forms, one definitely animal-like, the other plant-like.

The bacteria-free strain of *Colpidium striatum* employed was the one used in a previous investigation (Elliott, 1933). The pure strain of *Hæmatococcus pluvialis* (bacteria-free) was originally obtained from Professor E. G. Pringsheim, and was used recently in a morphological study (Elliott, 1934). Professor R. J. Williams very kindly supplied the pantothenic acid in the form of a calcium salt which had a potency of " 244 "; " one milligram added to 2.5 liters of culture medium gave a heavy response with yeast in 18 hours." Difco tryptone was employed in an organic basic culture medium, which was made up as follows:

Difco tryptone	10.0 gram
KH_2PO_4	2.0 "
Distilled water	1.0 liter

This medium was used throughout the experiments with *C. striatum*, while with *H. pluvialis* the tryptone content was reduced to 0.5 per cent because previous experiments had shown that the flagellate grew better in such a concentration.

For experimental purposes large quantities of the basic medium were made up and subsequently divided into two parts; to one portion was added HCl in titrating for the lower pH values and NaOH was added to the other portion in titrating for the higher pH values. Usually 10 tubes were set at each desired pH unit, each one of which contained 8 cc. of basic medium; to 5 of these (after sterilization) 1.0 cc. of a sterile pantothenic acid solution (10 mg. in 250 cc. of distilled water) was added. To the other 5 tubes distilled water (1 cc.) was added. The tubes were then inoculated with organisms from a stock culture prepared in the following manner. A heavily growing culture tube of the organisms was pipetted into a sterile centrifuge tube and centrifugalized. This was repeated at least four times with sterile tap water. Finally the washed organisms were pipetted into a dilution flask containing 200 cc. of sterile tap water. From this flask 1-cc. inoculations were made into each tube; the flask was vigorously shaken before each inoculation in order to insure an even distribution of the organisms. One tube was then selected from each set and tested for the initial pH. Most of the series were incubated at room temperature (19–26° C.) for a period of 76 hours or longer. After the incubation period was completed, two or more of the tubes from each set were checked for the final pH. One-half cc. of Bouin's fixative was then added to these and the remaining tubes and the final counts made; this was done with the aid of a Sedgwick-Rafter counting chamber and a Whipple ocular micrometer. A LaMotte roulette comparator was employed in making pH determinations.

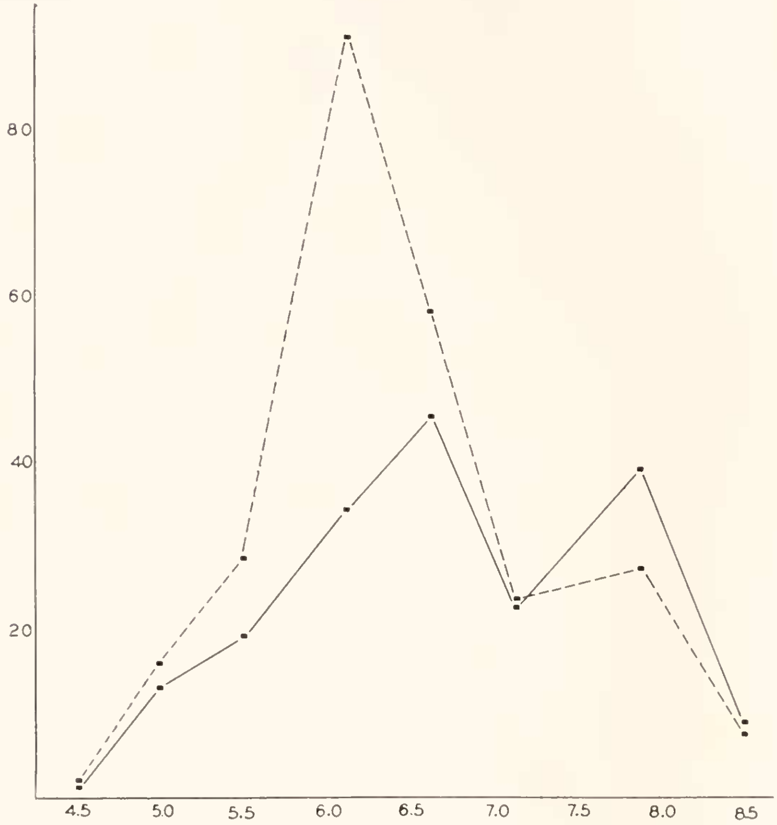

FIG. 1. Series I. Concentration of ciliates in thousands per cubic centimeter plotted against initial pH. The solid line indicates growth in the control; the broken line, growth with pantothenic acid.

EXPERIMENTAL

In testing the effect of any ionizable substance such as pantothenic acid, it is essential to determine its influence over a wide pH range, and not at the point of optimum growth alone of the organism. A substance may have decidedly different effects in the ionized and molecular condition. This fact was well illustrated in the case of acetic and butyric acid (Elliott, 1933a); these two substances were definitely toxic in the lower pH ranges (6.5–4.5), while above pH 7.0 they actually accelerated growth of *Colpidium striatum* and *C. campylum*. For this reason pantothenic acid was tested over a wide pH range.

Series I—Colpidium striatum

The pH values were set at 4.5, 5.0, 5.5, 6.3, 6.6, 7.2, 7.9, and 8.5. After sterilization and inoculation the pH remained the same in all the tubes. After 80 hours of incubation at room temperature there were no pH changes, except in the tubes set at pH 6.3 and there the change was within the experimental error of the readings (± 0.1). The final counts are recorded in Fig. 1.

It is obvious that a marked acceleration occurred in the tubes containing pantothenic acid over those of the controls. A noticeable increase was observed at pH 5.5, with a maximum at 6.3 and 6.6; there was no traceable increase at 7.2 and actual deceleration above this point. Pantothenic acid, as evidenced by this series, accelerates growth of *Colpidium striatum* in the acid range but has no effect in the alkaline range. The bimaximal pH growth curve in the control is typical for this species (Elliott, 1933a).

This entire series was duplicated (Series Ia) with similar results.

Series II—Hæmatococcus pluvialis

The experimental procedure in this series was very similar to that in Series I. The tubes were set at the following pH units: 4.5, 5.0, 5.5, 5.9, 6.5, 7.0, 7.5, 8.2, and 8.5. After 96 hours of incubation at room temperatures and north window illumination there were no pH shifts. The final counts are recorded in Fig. 2.

The results indicate that pantothenic acid had no effect whatever on the growth of *Hæmatococcus pluvialis* over the entire pH range. The differences at pH 7.0 and 8.2 are probably insignificant. It appears then that this substance accelerates the growth of a saprozoic ciliate, yet has no effect on a chlorophyll-bearing flagellate.

Series III—Colpidium striatum

It was shown in a previous investigation (Elliott, 1933b) that gelatin would not support growth of *Colpidium* beyond the third transfer. Gelatin lacks certain amino acids (tryptophane, isoleucine, and hydroxy glutamic acid) but is rich in lysine which is an essential amino acid for the growth of this organism (Hall and Elliott, MSS). Some other substance is apparently lacking in this protein. It occurred to the writer that perhaps pantothenic acid might be such an essential missing substance. For that reason it was thought worth while to determine the effect of pantothenic acid on the growth of *Colpidium striatum* when gelatin was employed as a basic protein. The experimental procedure was very similar in this series to that in the previous two with the exception that Difco gelatin (1 per cent solution) was substituted for Difco tryptone in the basic medium. The pH was adjusted as before at the following values: 4.5, 5.5, 6.5, 7.0, 7.5, 8.0, and 8.5. Pantothenic acid was added to one half of the tubes, sterilized, inoculated, incubated and counted. The final determinations are recorded in Fig. 3.

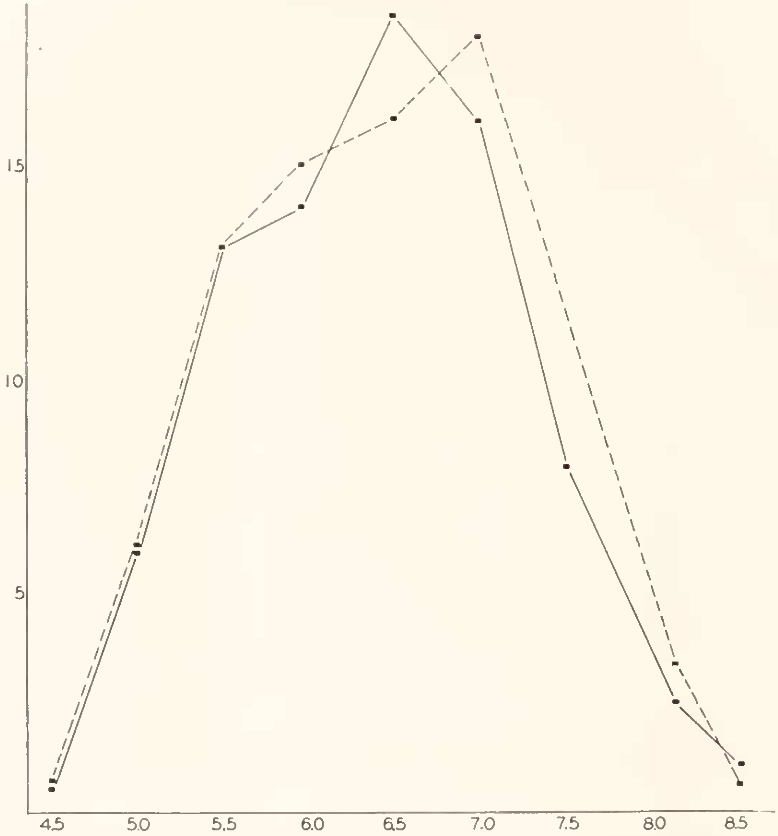

FIG. 2. Series II. Concentration of flagellates in thousands per cubic centimeter plotted against initial pH. The solid line indicates growth in the control; the broken line, growth with pantothenic acid.

The slight growth which occurred in the control tubes was probably due to substances carried over with the original inocula; the stock organisms were not washed as in the previous series. For that reason a small amount of growth would be expected. Had the washings and dilutions been made, no growth would probably have resulted. The essential point, however, is that pantothenic acid is apparently not the substance lacking in gelatin for the support of the growth of *Colpidium*.

Some other, still unknown, substance is deficient in this incomplete protein.

Series IV—Colpidium striatum

It was shown in a previous investigation (Hall and Elliott, MSS) that by adding small quantities of yeast extract to gliadin that growth could be maintained indefinitely; likewise, by adding a small amount of yeast extract to zein, together with lysine which is lacking in this protein, indefinite growth could be maintained. This indicated that some substance in yeast was essential for the growth of *Colpidium*. Perhaps this substance was pantothenic acid? The following series of experiments was devised to test this point.

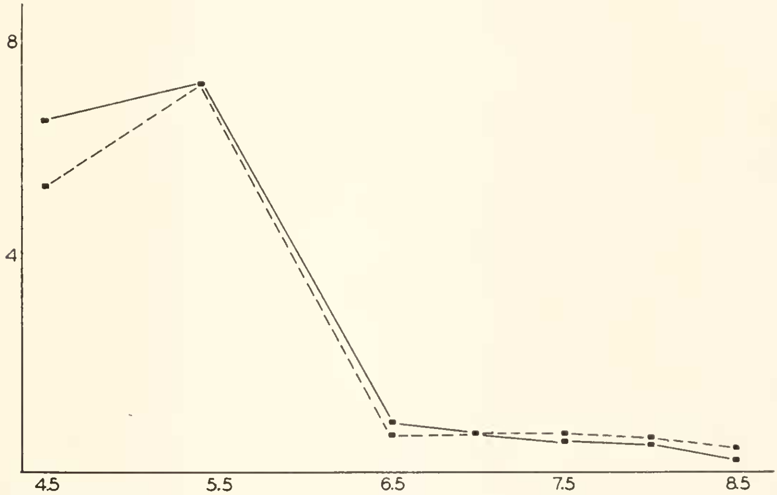

Fig. 3. Series III. Concentration of ciliates in thousands per cubic centimeter plotted against pH. The solid line indicates growth in the gelatin control; the broken line, growth with pantothenic acid.

The procedure was slightly different from that employed in the foregoing series. The basic medium consisted of 1 per cent protein (zein, gliadin, or gelatin) and 0.2 per cent KH_2PO_4 in distilled water. The pH was set at 5.8 in all tubes since previous experiments had demonstrated that acceleration occurred in that range. To 6 tubes containing zein (8 cc. each) lysine was added (1 cc. of 0.7 per cent stock solution); to each of 6 others, was added 1 cc. of pantothenic acid (concentration identical with that used in Series I); and to a third set of 6 tubes was added both lysine and pantothenic acid. In a similar manner lysine and pantothenic acid were added to 3 sets (6 each) of tubes containing gliadin. In this case, however, lysine was added because the concentration of this amino acid in gliadin is low (0.92 per cent). Gelatin was included in this series merely as a check on the previous

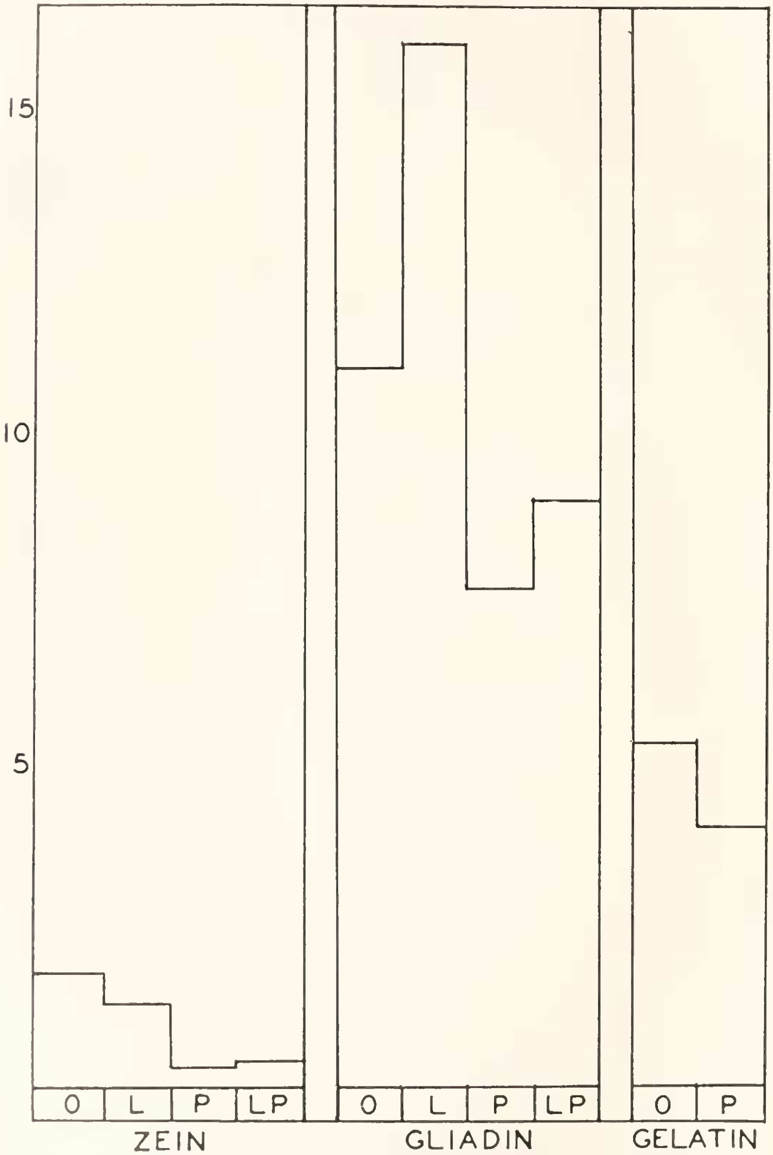

FIG. 4. Series IV. Concentration of ciliates in thousands per cubic centimeter plotted on ordinate. The letters in each column indicate substances added: *O*, nothing; *L*, lysine; *P*, pantothenic acid; *LP*, both lysine and pantothenic acid.

observations. All the tubes were inoculated from a stock culture which had not been washed so that a small amount of the original medium was carried over into each tube. This would permit a small amount of growth even if the tested substance was inadequate to support continued growth. The incubation period was 80 hours and the final counts are recorded in Fig. 4.

It is observed at once that in the case of all three substances, zein, gliadin, and gelatin, pantothenic acid had no accelerating effect. In fact, an actual deceleration is noted in all three cases, although perhaps significant only in the case of gliadin. The growth was comparatively slight in all tubes, which was to be expected if pantothenic acid was ineffective. It may be concluded, therefore, that pantothenic acid is not the substance essential for life of *Colpidium* which is lacking in zein, gliadin, and gelatin.

Discussion

The question of growth-promoting substances in microörganisms has commanded considerable attention for many years. It was initiated by Wildiers (1901), who isolated his so-called "bios" from yeast, and who maintained that this substance was essential for continued growth of the organism. An excellent review of the history of the study of bios is given by Tanner (1925). Fulmer and Nelson (1922) recognized the significant increase of yeast growth by bios but found that it is not necessary for indefinite growth of the cells. Williams (1919) began a long series of experiments which finally resulted in his isolation of pantothenic acid (Williams et al., 1933) which he and his associates claim is identical with, or very closely related to, vitamin G (B_2). Several of its properties suggest such a relationship. It probably is one of the substances originally contained in Wildier's bios. Williams believes that the accelerating substance in the growth of yeast is a single substance, namely, pantothenic acid. Richards (1932), on the other hand, believes that thallium, found as an impurity in asparagin and many other sources, may be the element responsible for acceleration when used in proper concentrations. The striking effect of pantothenic acid on the growth of *Saccharomyces cerevisiæ,* as demonstrated by Williams and his associates (1933), seems more impressive than the results obtained by Richards with thallium. The former workers showed that concentrations as low as 0.02 mg. in 1 cc. of their synthetic culture medium was sufficient to accelerate the growth five times that of the control. Increasing concentrations produced corresponding increases in the growth rate; when the concentration reached 40.0 mg. per cc. of culture medium the fold increase was about 1500 times that of the control. The very high dilutions preclude the possibility of pantothenic acid being utilized as a source of food. Therefore, its activity in the organism probably simulates that of vitamins in higher forms.

Although the effect on Protozoa is not so striking as that produced with yeast, nevertheless the present experiments indicate that the effect of pantothenic acid on the growth of *Colpidium striatum* at certain pH values is very pronounced. The increase is definite on the acid side of neutrality, while above pH 7.0 no acceleration occurred. It was at first thought that this might be due to the toxic effect of the dissociated acid (ionization constant is approximately 3.9×10^{-5} according to Williams and Moser, 1934). However, upon examination of the properties of the acid, it was found that according to Williams it is thermo-labile in the alkaline range. They say, "It is evident that nearly all of its (pantothenic acid) activity . . . is destroyed by prolonged heating in weakly alkaline medium but that heating in weakly acid or neutral medium for 4 hours at 119° C. causes very little destruction." The destruction of its accelerating powers on the alkaline side in Series I might have been due to the heating to 122° C. for 20 minutes when the tubes were sterilized.

The question of synthesis of pantothenic acid by plants and animals was studied by Williams and his associates (Williams et al., 1933). They found that *Aspergillus niger* synthesized the acid in soils and this led them to believe that it was not produced by green plants. They say, "that it is without doubt produced by microörganisms in soil suggests the possibility that it may not be synthesized by green plants." This prediction is of interest in view of the present observations. It was noted that the green phytomonad, *Hæmatococcus pluvialis*, failed to show any acceleration with pantothenic acid while a marked increase was noted with the saprozoöic organism, *Colpidium striatum*. This does not prove that *Hæmatococcus pluvialis* fails to synthesize the acid but it does seem to indicate that the organism does not utilize it to any great extent.

It is not at all unusual that microörganisms are able to synthesize vitamins; for example, Kuroya and Hosaya (1923) showed that *Bacterium coli* synthesized vitamin B, and Damon (1924) demonstrated that several members of the genus *Mycobacterium* produced a growth-stimulating substance analogous to vitamin B. It appears that this ability to synthesize vitamin B is widespread among bacteria and yeasts. Vitamin G (B_2) is probably synthesized along with B since it has somewhat similar distribution and properties. Both vitamin B and G were probably present in the Difco tryptone base (partially hydrolized casein) used in the present experiments, but since all the tubes were autoclaved at one time or another, properties of G were undoubtedly impaired. Furthermore, the writer has maintained bacteria-free cultures of Protozoa for several years in autoclaved media. Since vitamin B is destroyed by such treatment, it is probably not essential for the growth of these organisms. On the other hand, vitamin G (B_2), being heat stable under most conditions, probably was retained unharmed for the most part and possibly utilized by the ciliates. If vitamin G and pantothenic acid prove to be identical substances the results of the present investigation will carry more interest. While these observations do not demonstrate the essential nature of pantothenic acid in regard to continued culturing of *Colpidium siriatum*, they do indicate its importance in rate of multiplication of the ciliate.

Summary

Pantothenic acid, a growth-promoting substance of universal occurrence, was tested for its effect on the growth of two remotely related protozoan forms, namely, *Colpidium striatum* and *Hæmatococcus pluvialis*. The tests were conducted over a wide pH range. In the case of *C. striatum* a doubling of the growth occurred in the pantothenic acid cultures on the acid side of neutrality (pH 5.5–6.6) while no acceleration was observed above pH 7.0. With *Hæmatococcus pluvialis* no differences were noted in the test and the control tubes. It was shown further that pantothenic acid was not the substance lacking in certain incomplete proteins (zein, gliadin, and gelatin) which failed to support indefinite growth of *C. striatum*.

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